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Essential Role for *Sonic hedgehog* during Hair Follicle Morphogenesis

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The hair follicle is a source of epithelial stem cells and site of origin for several types of skin tumors. Although it is clear that follicles arise by way of a series of inductive tissue interactions, identification of the signaling molecules driving this process remains a major challenge in skin biology. In this study we report an obligatory role for the secreted morphogen Sonic hedgehog (*Shh*) during hair follicle development. Hair germs comprising epidermal placodes and associated dermal condensates were detected in both control and *Shh* $-/-$ embryos, but progression through subsequent stages of follicle development was blocked in mutant skin. The expression of *Gli1* and *Ptc1* was reduced in *Shh* $-/-$ dermal condensates and they failed to evolve into hair follicle papillae, suggesting that the adjacent mesenchyme is a critical target for placode-derived *Shh*. Despite the profound inhibition of hair follicle morphogenesis, late-stage follicle differentiation markers were detected in *Shh* $-/-$ skin grafts, as well as cultured vibrissa explants treated with cyclopamine to block *Shh* signaling. Our findings reveal an essential role for *Shh* during hair follicle morphogenesis, where it is required for normal advancement beyond the hair germ stage of development. © 1999 Academic Press

Key Words: hair follicle; Sonic hedgehog; morphogenesis; cyclopamine; vibrissae.

INTRODUCTION

Early stages of organogenesis are marked by the appearance of mesenchymal condensates and focal cellular aggregates, or placodes, in adjacent epithelia. This process is driven to completion by a series of inductive signals traveling between epithelial and mesenchymal cell populations which ultimately give rise to the adult structure (reviewed

in Gurdon, 1992; Thesleff *et al.*, 1995). In skin appendages such as vibrissae and hair follicles, detailed analysis of tissue recombinants has revealed the existence of at least three morphogenetic signals: the embryonic dermis instructs overlying ectoderm to initiate placode formation; the placode transmits a signal generating a dermal condensate with hair follicle-inductive properties; and the condensate in turn sends a signal to nascent follicle keratinocytes stimulating their proliferation, downgrowth into the developing dermis, and reorganization to form the mature follicle (reviewed in Sengel, 1976; Hardy, 1992). The epithelial and mesenchymal components of the follicle remain in close proximity in mature hair bulbs, where the dermal papilla is surrounded by matrix cells giving rise to at least six

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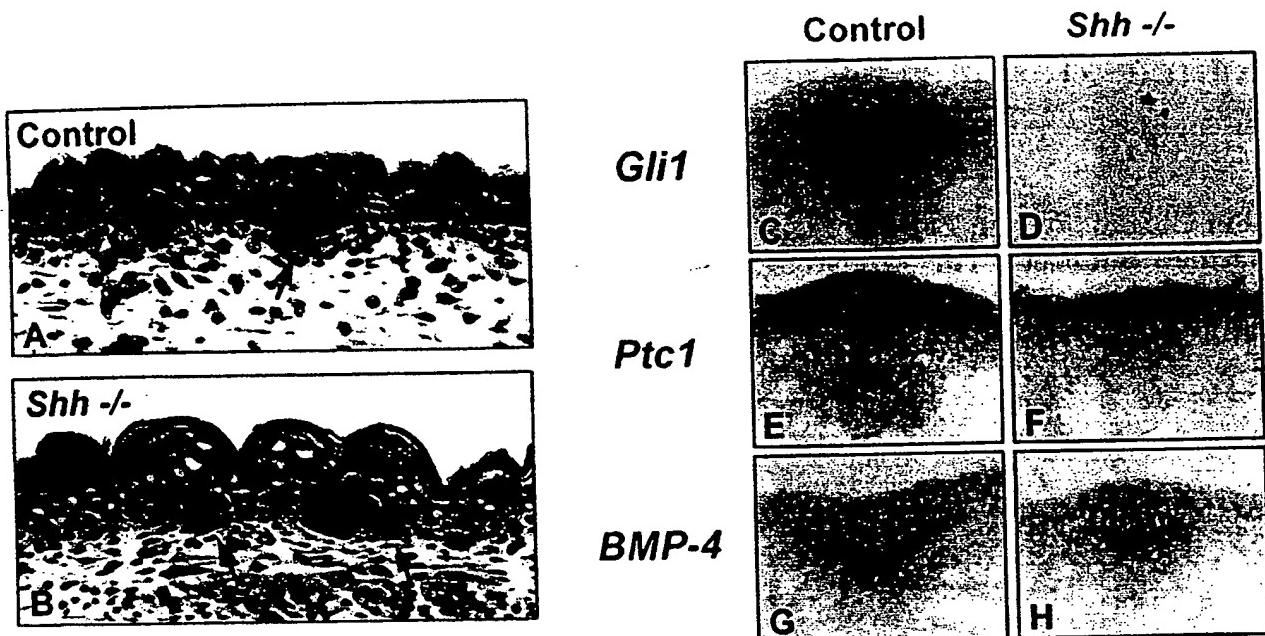


FIG. 1. Morphology and gene expression patterns of control and *Shh* $-/-$ primary hair germs. [A, B] Normal-appearing hair germs consisting of an epithelial placode and adjacent mesenchymal condensate (arrows) were detected in skin of both control [A] and *Shh* $-/-$ [B] embryos at 15.5 days of gestation [H & E staining]. [C-H] Altered abundance of Shh target genes in hair germs in *Shh* $-/-$ mouse skin. Expression of *Gli1* [C, D], *Ptc1* [E, F], and *BMP-4* [G, H] transcripts was examined in E15.5 mouse skin using digoxigenin-labeled cRNA probes. Note virtual absence of *Gli1* in both epithelial and mesenchymal components of the mutant hair germ and reduced mesenchymal *Ptc1* expression in *Shh* $-/-$ skin.

phenotypically distinct epithelial cell types in the hair shaft and inner root sheath of the hair follicle. After birth the follicle epithelium cycles through periods of active growth (anagen), followed by regression (catagen) and inactivity (telogen) [reviewed in Cotsarelis, 1997]. The morphogenetic program that accompanies the transition from telogen to anagen bears similarities to follicle development during embryogenesis, making this structure a unique model for studying certain aspects of organogenesis in the adult animal. Although a large number of genes have been implicated at various stages of hair follicle development and cycling [reviewed in Rosenquist and Martin, 1996; Stenn *et al.*, 1996; Widelitz *et al.*, 1997; Millar, 1997], the molecular nature of the inductive signals that underlie the formation of the follicle is largely unknown.

In situ localization of transcripts encoding potential morphogens has revealed focal expression of *Sonic hedgehog* (*Shh*) in placodes of the epidermis and several other epithelia at early stages of development, with *Ptc1* transcripts encoding a putative Shh receptor also present in adjacent mesenchymal cells (Bitgood and McMahon, 1995; Iseki *et al.*, 1996; Oro *et al.*, 1997; Motoyama *et al.*, 1998). These findings, coupled with the accumulating evidence demonstrating a pivotal role for secreted Hedgehog proteins

in a variety of developmental processes [reviewed in Hammerschmidt *et al.*, 1997], led us to examine the potential involvement of this pathway in hair follicle morphogenesis. Since the follicle is a source of cutaneous stem cells and a likely site of origin for certain epithelial skin cancers (Cotsarelis *et al.*, 1990; Lavker *et al.*, 1993; Rochat *et al.*, 1994; Hansen and Tennant, 1994), understanding the developmental biology of this organ is likely to provide insights relevant to normal skin function, as well as wound healing and neoplasia, and may shed light on fundamental aspects of organogenesis involving other structures as well.

METHODS

Animals and Skin Transplantation

The generation and identification of *Shh* mutant mice were performed as described [Chiang *et al.*, 1996]. Embryonic skin was grafted onto the dorsal fascia of nude mice beneath a protective silicone chamber using a modification of a previously described technique (Dlugosz *et al.*, 1995). The chamber was removed 11–12 days after grafting and tissue harvested for analysis after an additional 1 to 4 weeks. Animals were handled in accordance with NIH guidelines.

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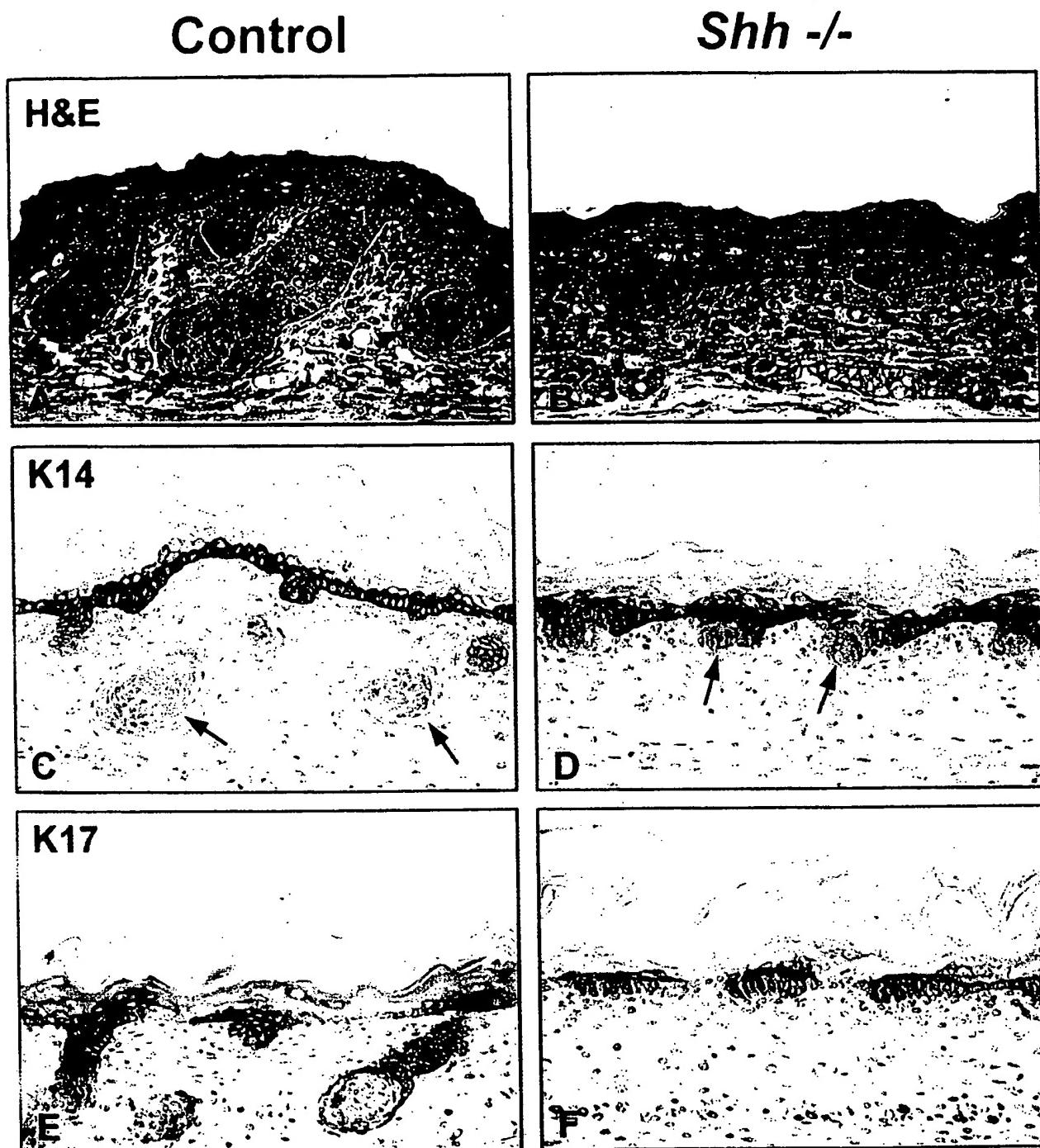


FIG. 2. Inhibition of hair follicle morphogenesis, but not biochemical differentiation, in *Shh* -/- mouse skin. (A, B) Advanced hair follicle development in skin from control (A) but not *Shh* -/- (B) embryos at 17.5 days of gestation [H & E staining]. Note the dermal papilla (arrow) surrounded by an epithelial bulb of the largest hair follicle and organizing mesenchymal aggregates (arrowheads) adjacent to invaginating tips of less mature follicles (A). In striking contrast, dermal papillae are not detected in *Shh* mutant skin (B). (C-F) Immunohistochemistry revealing similar patterns of keratin expression in control and *Shh*-deficient follicles. Absence of keratin K14 immunostaining in a subpopulation of keratinocytes in both control (C) and *Shh* -/- (D) hair follicles (arrows). Induction of nonepidermal keratin K17 in hair follicle keratinocytes in control (E) and *Shh* mutant (F) skin.

Immunohistochemistry

Tissue was fixed overnight in Carnoy's or Bouin's solution for detecting keratins (K1, K10, K5, K14, and K17), loricrin, and filaggrin; fixation with neutral-buffered formalin was used for tissues immunostained with Lef-1, Ki67, and hair keratin (AE13) antibodies. Samples were embedded in paraffin and 8- μm sections cut for immunostaining. Immunoreactivity of antigens in formalin-fixed sections was restored by immersing slides in a boiling 0.01 M citrate buffer, pH ~6, for 10 min. The following primary antibodies were used at the indicated dilutions for immunostaining: rabbit anti-keratins K1, K10, K5, and K14 (1:500) [Roop et al., 1984] and loricrin and filaggrin (1:500) [Roop et al., 1987], supplied by Dr. Stuart Yuspa; rabbit anti-K17 (1:1000) [McGowan and Coulombe, 1998], provided by Dr. Pierre Coulombe; rabbit anti-Lef-1 (1:200) [Travis et al., 1991], a gift from Dr. Rudolf Grosschedl; rabbit anti-Ki67 and NCL-Ki67p [Novocastra Laboratories, Ltd., Newcastle upon Tyne, UK] (1:200); and mouse monoclonal AE13 hybridoma supernatant, which recognizes type I hair keratins (1:5) [Lynch et al., 1986], provided by Dr. Tung-Tien Sun. Tissue sections were incubated with primary antibodies diluted in Tris-buffered saline containing 1% bovine serum albumin, typically for 1–2 h at room temperature. Subsequent immunostaining procedures were performed using peroxidase Vectastain ABC kits [Vector Laboratories, Inc., Burlingame, CA] and 3,3'-diaminobenzidine (Sigma, St. Louis, MO) as a substrate, according to the manufacturers' recommendations. Sections were counterstained with hematoxylin and mounted using Permount (Fisher Scientific, Pittsburgh, PA).

In Situ Hybridization

Non-radioactive RNA *in situ* hybridization was performed on 5- μm sections essentially as described [Groves et al., 1995], using previously described sequences for *Gli1* [Walterhouse et al., 1993], *Ptc1* [Goodrich et al., 1996], and *BMP-4* [Jones et al., 1991].

Vibrissa Follicle Explants

Vibrissa follicle explants were established using CD-1 mouse embryos at 13.5 days of gestation according to a previously described protocol [Hirai et al., 1989], with minor modifications. Vibrissa pads were transferred onto Nuclepore filters (13 mm, 8- μm pores) and floated on 2 ml of medium [DMEM (Life Technologies, Gaithersburg, MD) + Ham's F12 medium (Life Technologies) (1:1), with 1% FCS (Intergen, Purchase, NY), penicillin (50 units/ml), and streptomycin (50 $\mu\text{g}/\text{ml}$) (Life Technologies)] in six-well plates. Similar results were obtained using a DMEM-based medium, without the addition of Ham's F12. Explants were fed fresh medium every 2 days. Microdissection was performed with the aid of a Nikon SMZ-2T stereomicroscope and photomicrographs were taken using an Olympus OM-4 camera. Cyclopamine was stored at -20°C as a 10 mM stock in 95% EtOH.

RNA Isolation and RT-PCR

RNA was obtained by solubilizing individual explants in TriZol (Life Sciences) and isolating as recommended by the manufacturer. cDNA was synthesized using SuperScript II RNase H reverse transcriptase with random primers (Life Technologies), and RT-PCR was performed using the following primers: MHKA1 (318-bp

product) [forward, 5'-ATCAGAGAATGCCAGGTTGG-3'; and reverse, 5'-TCATTGAGCACACGGTTCAAG-3']; hacl-1 (308-bp product) [forward, 5'-TTGTATCTCCACTCCTGCC-3'; and reverse, 5'-AGACTCCACAGGGTTGG-3']; profilaggrin (330-bp product) [forward, 5'-GCTTAAATGCATCTCCAG-3'; and reverse, 5'-AGTCAGTCCTATTCCAGG-3'] [Bickenbach et al., 1995]; β actin (421-bp product) [forward, 5'-TACACAGGCATTGTGATGG-3'; and reverse, 5'-CAACGTCACACTTCATGATGG-3'] [Walterhouse et al., 1993]. The following PCR conditions were used for MHKA1, Hacl-1, and β actin: 95°C × 3 min "hot start"; 95°C × 50 s, 58°C × 30 s, and 72°C × 60 s for 25 (β actin) or 35 cycles (MHKA1 and Hacl-1); and 72°C × 7 min. PCR conditions for profilaggrin primers were as previously described [Bickenbach et al., 1995]. Reaction products were run through 1.5% agarose gels and visualized with ethidium bromide.

RESULTS AND DISCUSSION

Early stages of hair follicle development appeared similar in control and *Shh* -/- embryos. Hair germs, consisting of clusters of columnar basal keratinocytes protruding into the developing dermis with associated dermal condensates, were detected in the skin of both mutant and control embryos at 15.5 days of gestation [Figs. 1A and 1B]. Despite the similar morphology of control and *Shh*-deficient hair germs, a dramatic difference in gene expression patterns was revealed by *in situ* hybridization. The level of *Gli1* mRNA was markedly reduced in both the epithelial and mesenchymal components of *Shh* -/- primary hair germs [Figs. 1C and 1D]. In addition, expression of *Ptc1* was reduced in *Shh* mutant hair germs, although some placodes contained levels slightly above background [Figs. 1E and 1F]. These findings are consistent with previous reports identifying *Shh* as a positive regulator of both *Gli1* and *Ptc1* [Marigo and Tabin, 1996; Marigo et al., 1996; Lee et al., 1997; Sasaki et al., 1997] and suggest that *Shh* is signaling in both the epithelial and mesenchymal cells of the developing follicle. In contrast to *Gli1* and *Ptc1*, *BMP-4* mRNA was clearly detectable in condensates of mutant and control embryos [Figs. 1G and 1H], arguing against a requirement for *Shh* in the induction of *BMP-4* expression. Thus, although *Shh* is not required for the initiation of hair follicle development, primary hair germs that arise in *Shh* mutant skin are deficient in the expression of at least some *Shh* target genes.

In control embryos, the interval between E15.5 and E17.5 is marked by rapid proliferation and downgrowth of the follicle into the developing dermis, accompanied by a several-fold increase in the mass of the follicle epithelium and reorganization into distinct cellular compartments. In the most mature follicles, keratinocytes in the most peripheral cell layer, which give rise to the outer root sheath in the mature follicle, have assumed a columnar arrangement perpendicular to the long axis of the developing follicle; cells located centrally are without a definite orientation at this stage but will eventually be replaced by the three

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concentric layers of inner root sheath cells and the three cell types comprising the hair shaft; and the epithelial cells of the deepest portion of the follicle, the future hair bulb, have surrounded what is at this stage a well-defined cluster of mesenchymal cells, the dermal papilla (Fig. 2A, arrow). Even the less mature follicles exhibit an organized "cap" of mesenchymal cells at their invaginating tips (Fig. 2A, arrowheads). In striking contrast, hair follicles in skin from mutant embryos at E17.5 failed to develop past the hair germ stage seen at E15.5 (Fig. 2B). Although the follicle epithelium was most obviously affected due to its lack of growth, organizing dermal condensates and dermal papillae were conspicuously absent in mutant skin. These results are consistent with the idea that epidermis-derived Shh (Bitgood and McMahon, 1995; Iseki *et al.*, 1996; Oro *et al.*, 1997; Motoyama *et al.*, 1998) functions as a paracrine signal regulating development of the mesenchymal component of the hair follicle. Inhibition of follicle formation is not likely to be due to a general disruption of skin development since epidermal morphogenesis, marked by the appearance of granular and cornified cell layers, took place by E17.5 in both control and mutant embryos (Figs. 2A and 2B).

Additional studies were performed to determine whether Shh influenced the expression of epithelial differentiation markers in embryonic skin. Keratinocytes in developing hair follicles can be distinguished by a relative deficiency of K5 and K14, keratins that are abundant in surrounding epidermal basal cells (Kopan and Fuchs, 1989; Byrne *et al.*, 1994). Immunohistochemical staining of E17.5 embryos revealed greatly reduced or undetectable levels of K14 in a subpopulation of cells comprising the normal follicles in control embryos as well as the primordial follicles seen in *Shh* *-/-* embryos (Figs. 2C and 2D, arrows). Moreover, K17, which is normally not detected in interfollicular epidermis but is expressed in developing and mature hair follicles (Panteleyev *et al.*, 1997; McGowan and Coulombe, 1998), was localized to the follicular epithelium in both control and mutant skin (Figs. 2E and 2F). Thus, although morphogenesis of hair follicles in *Shh* *-/-* skin fails to progress past the hair germ stage, these structures contain epithelial cells that have initiated a terminal differentiation program characteristic of developing follicle keratinocytes. Consistent with the morphological findings in Figs. 2A and 2B, the expression level of epidermal-specific differentiation markers (keratins 1 and 10, loricrin, and filaggrin) in *Shh* *-/-* skin was similar to or greater than in control epidermis, based on immunohistochemical staining (data not shown).

Since *Shh* *-/-* mice are not viable, postnatal analysis of mutant skin was performed following grafting onto nude mice. Whereas skin from control mice produced abundant pigmented hairs, transplanted *Shh* *-/-* skin failed to generate detectable hairs but exhibited a pigmented graft site, consistent with the strain of donor skin (Fig. 3A). The histology of control skin grafts revealed the typical structures seen in normal mouse skin, including numerous hair

follicles and sebaceous glands (Fig. 3B). In striking contrast, mutant skin failed to produce normal-appearing follicles, hair shafts, or sebaceous glands, but in some cases (three of a total of seven *Shh* *-/-* grafts) exhibited a thickened epidermis with focal areas of hyperkeratosis (Fig. 3C). Conspicuous aggregates of basophilic cells with scant cytoplasm were detected at the dermal-epidermal junction in these mutant grafts (Fig. 3C, arrows). Interestingly, the morphology of cells in the *Shh*-deficient keratinocyte aggregates was reminiscent of cells in control hair bulbs, and additional analyses revealed biochemical similarities. Cells in these aggregates were unreactive with K5 antibodies (Fig. 3D, arrows), exhibited abundant nuclear Lef-1 expression (Fig. 3E) (Zhou *et al.*, 1995), and contained a high proportion of proliferating cells detected by Ki67 immunostaining (data not shown). Interestingly, short columnar structures resembling abortive hair shafts were associated with some of the *Shh* mutant keratinocyte aggregates. Moreover, these structures expressed hair-specific keratin (Fig. 3F), indicating that an advanced stage in the follicle differentiation program was achieved despite a dramatic disruption of normal morphogenesis. Rarely, a small cluster of mesenchymal cells was seen associated with the base of a keratinocyte aggregate, as illustrated in Fig. 3E, where these cells immunostain with Lef-1 antibody. These findings suggest that a rudimentary dermal papilla is present in at least some of the hair germs seen in *Shh* mutant grafts.

To better define the temporal requirement for Shh during follicle development, tissue culture studies were performed using cyclopamine (Gaffield and Keeler, 1996), which has recently been shown to block Shh signaling in neural plate explants (Cooper *et al.*, 1998; Incardona *et al.*, 1998). Explants were established using vibrissa pads obtained from mice at 13.5 days of gestation (Hirai *et al.*, 1989). When grown for 6 to 8 days in culture, explants undergo robust morphogenesis resulting in the formation of elongated, grossly normal-appearing vibrissa follicles (Fig. 4A). These follicles contained hair shafts and expressed genes encoding mouse hair keratin A1 (MHKA1) (Kaytes *et al.*, 1991) and a hair cortex-specific marker, Hacl-1 (Huh *et al.*, 1994), detected by RT-PCR (Fig. 4B). Treatment of explants with cyclopamine results in striking inhibition of morphogenesis, indicating that Shh signaling is required during or shortly after the hair germ stage of vibrissa follicle development (Fig. 4C). In keeping with our results obtained using *Shh* mutant skin, hair-specific transcripts are detected in cyclopamine-treated grafts (Fig. 4B) despite their altered development, providing further support for the notion that biochemical differentiation of the follicle is not necessarily coupled to its morphogenesis. Both control and cyclopamine-treated explants accumulate proflaggrin mRNA, indicating that disruption of Shh signaling does not inhibit epidermal differentiation.

Collectively, the results of our studies reveal an obligatory role for Shh in the progression of hair follicle

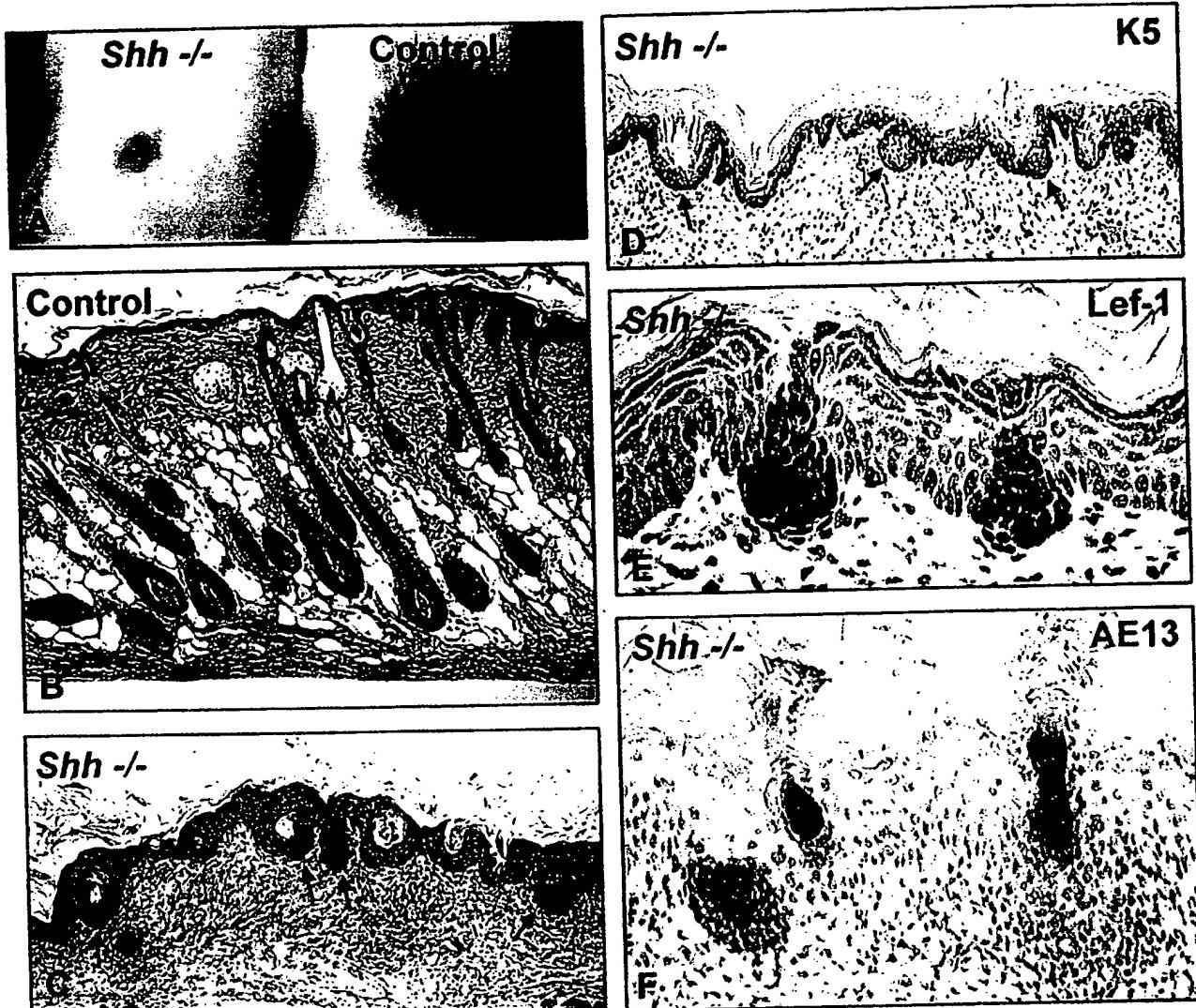


FIG. 3. Impaired hair follicle development in *Shh* mutant skin grafted onto nude mice. (A) Gross appearance of nude mouse graft sites 6 weeks after transplantation. Note robust hair growth in control graft compared to hairless, but pigmented, *Shh* $-/-$ skin graft. (B, C) H & E staining. Histologically normal-appearing skin in control graft (B) contains mature hair follicles with associated sebaceous glands and subcutaneous adipose tissue. Abnormal skin development in *Shh* $-/-$ graft characterized by a thickened epidermis containing keratinocyte aggregates (arrows) at the base of the epidermis (C). (D-F) Immunohistochemistry. Unlike adjacent epidermal cells, *Shh* $-/-$ keratinocyte aggregates do not express K5 (D, arrows) but are positive for Lef-1 localized to nuclei (E). Note also the presence of a small cluster of Lef-1 positive mesenchymal cells associated with the keratinocyte aggregate on the right (E). Immunostaining of abortive hair shafts with hair-specific keratin antibody AE13 (F), revealing an advanced stage of follicle differentiation in *Shh* mutant skin.

morphogenesis past the hair germ stage of development. Both the reduced expression of *Ptc1* and *Gli1* in *Shh* $-/-$ dermal condensates, and their failure to evolve into recognizable dermal papillae, argue that *Shh* is involved in regulating development of the mesenchymal component of the hair follicle, although a requirement for *Shh*

signaling in the epithelial component of the follicle cannot be excluded. In the absence of dermal papillae, normal hair follicle morphogenesis does not proceed, underscoring the critical influence these cells have on growth and remodeling of the developing follicle epithelium [Jahoda *et al.*, 1984; Weinberg *et al.*, 1993].

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FIG. 4. Membrane markers when fibroblasts are product of *Shh* signaling.

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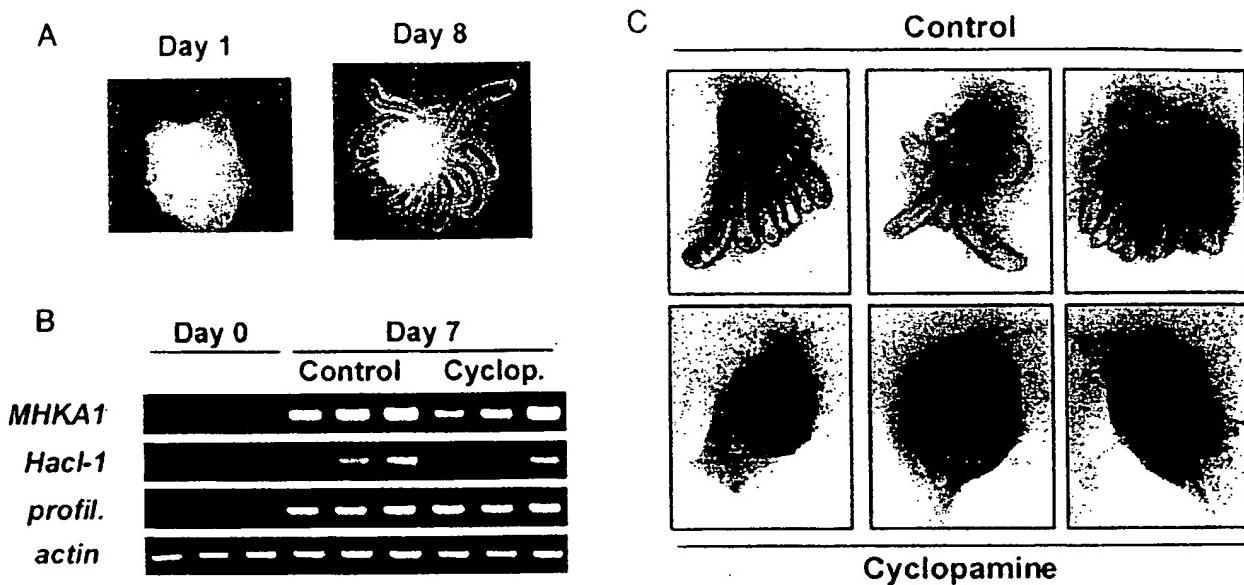


FIG. 4. Cyclopamine impairs vibrissa follicle morphogenesis in explant cultures. [A] Vibrissa pad explants growing on Nuclepore membranes on day 1 and day 8 in culture (dark-field). [B] RT-PCR analysis examining expression of transcripts encoding hair-specific markers MHKA1 and HacI-1 and an epidermal differentiation marker filaggrin (*profil.*). RNA was obtained from embryonic vibrissa pads when first isolated (day 0) and after growth as explants (day 7) in the presence or absence of 1 μ M cyclopamine. Each lane contains reaction products for RNA isolated from an individual vibrissa pad. [C] Morphogenesis of vibrissa follicles is blocked by cyclopamine, an inhibitor of Shh signaling. Cyclopamine was present in the medium for the duration of the experiment.

Interestingly, biochemical differentiation of the follicle can take place in the absence of normal morphogenesis, implying that these two processes are regulated independently in this organ. Additional experiments will be required to formally define which component of the developing follicle is functionally impaired in *Shh* $-/-$ embryos and to determine whether Shh has additional roles at later stages of follicle development or during hair cycling. We anticipate that these studies may ultimately help explain how constitutive activation of the Shh signaling pathway in keratinocytes contributes to the formation of basal cell carcinoma (Johnson *et al.*, 1996; Hahn *et al.*, 1996; Oro *et al.*, 1997; Fan *et al.*, 1997; Xie *et al.*, 1998).

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